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13. ABSTRACT ***(Maximum 200 Words)***

We purposed to characterize a regulatory domain on p53 and identify kinase(s) that may be involved in the regulation. Since 7/1/97, we constructed a set of deletions to disrupt the potential regulatory domains of p53 on Gal4-p53(1-160) and on full-length p53. Using these mutants we have obtained the following results:

1. Mutants Gal4-p53N(Δ92-109), Gal4-p53N(Δ116-127) and Gal4-p53N(Ser116/127Ala) had little effect on the putative inhibitory effect.
2. TPA stimulation inhibited the transcriptional activities of Gal4-p53N(Δ92-109) and Gal4-p53N(Δ116-127), but did not affect Gal4-p53(1-92).
3. Mutants p53(Δ92-109), p53(Δ116-127), and p53(S116/127D) significantly lost their ability to activate transcription in vivo.
4. Mutants p53(Δ92-109), p53(Δ116-127) and p53(S116/127A) retained their abilities to bind to DNA, but p53(S116/127D) did not.
5. MAP-kinase is involved in the degradation of mutant p53 protein.

In conclusion, we have shown that two serine residues 116 and 127 may play a role in stabilization of p53 protein levels. Further experiments will be conducted to confirm these results. We have also showed that MAP-kinase is involved in the degradation of mutant p53 protein. Our findings suggest a role for MAPK in the degradation of mutated form of p53 protein and in cell differentiation. These results were published as a JBC paper

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INTRODUCTION

A putative domain may be present within p53 involving its regulation. Further evidence of a regulatory domain is derived from a comparison of p53 with two oncogenes, c-jun and c-fos. Amino acids 93-160 from p53 show a significant homology to c-jun and c-fos inhibitory domains, especially to c-jun's δ region. The c-jun δ region has been found to play an inhibitory role by interacting with a member of the MAPK family, JNK. Once bound to c-jun, JNK is capable of phosphorylating serine residues 63 and 73. Two conserved serine residues within p53's potential regulatory domain may also be involved in phosphorylation and regulation. Therefore, we hypothesized that p53 serine residues 116 and 127, homologous to c-jun serine residues 63 and 73, may be phosphorylated by JNK or another MAPK family member. Specifically, we proposed:

I. Construct Gal4-p53(Δ92-109) and Gal4-p53(Δ116-127) and study their transcription activities using a transient transfection assay.

II. Assay if JNK or another MAPK family member is involved in regulating p53 transcription activity.

III. Construct point mutants on serine residues 116 and 127.

IV. Study phosphorylation of p53 regulatory domain by JNK or other potential MAPK family member kinase(s).

BACKGROUND

p53 exerts its tumor suppression function by inducing growth arrest and apoptosis. The biochemical activity of p53 that is required for this relies on its ability to bind to specific DNA sequences and to function as a transcription factor. The importance of the activation of transcription by p53 is underscored by the fact that the majority of p53 mutations found in tumors are located within the domain required for sequence-specific DNA binding. Therefore, it is clear that this activity is critical to the role of p53 in tumor suppression.

Previous work by our group suggests a possible regulatory domain may be present within p53. Supporting preliminary data used Gal4-p53 constructs for deletion analysis of p53's transcription activity. Based on transient transfection assays, the region of p53 spanning residues 1-92 shows high transactivation activity. Extension of the transcriptionally active residues 1-92 with residues 93 to 160 resulted in a reduction in transcription by a factor of 100.

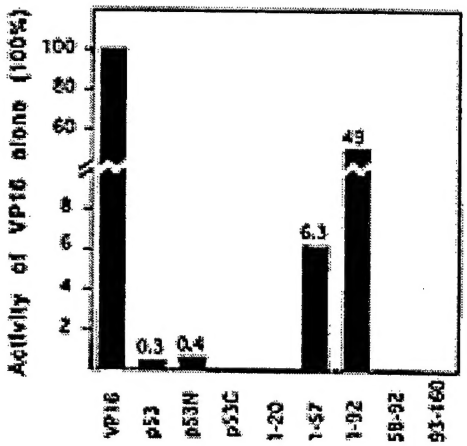
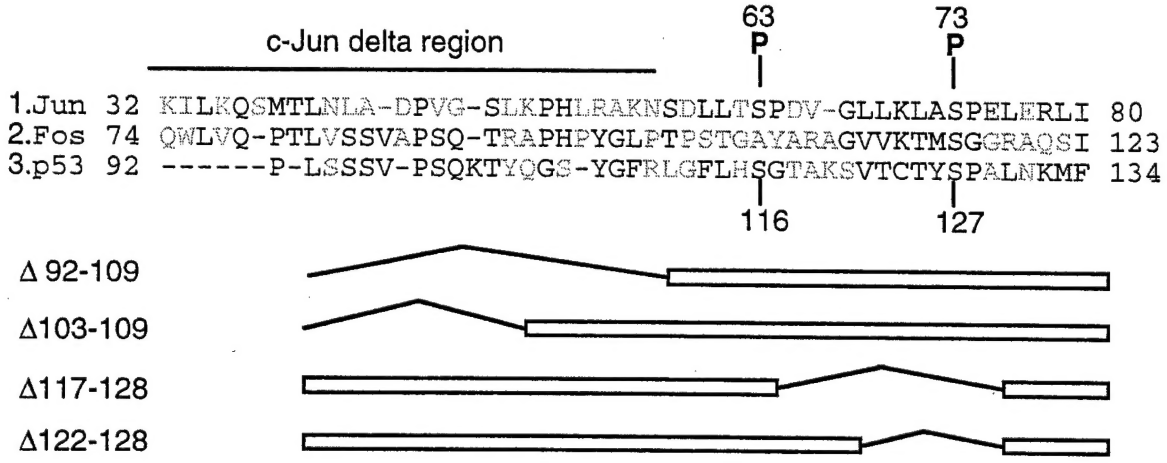


Figure 1. p53 contains an inhibitory domain. *In vivo* transactivation of G5E1B CAT gene by Gal4-p53 fusion proteins in transient transfection assays. CAT activity quantified with an AMBIS b-scanning system is represented as a percentage of the activity with Gal4-VP16.

Similar results have been observed in the c-jun and c-fos proteins, both of which contain conserved inhibitory domains capable of silencing their activation domains. Furthermore, the δ region of the c-jun inhibitory domain has been shown to interact with the c-jun N-terminal kinase (JNK). JNK is a member of the mitogenic activated protein kinase (MAPK) family which can

positively regulate c-jun activity through phosphorylation of serine residues 63 and 73. A comparison of the regulatory regions of c-jun and c-fos has identified homology with a p53 inhibitory domain (92-160, see below). It is interesting to note that the proposed p53 regulatory region also contains two conserved serine residues at position 116 and 127 which may also be phosphorylated by JNK or a JNK related kinase. Therefore, we proposed to characterize JNK or a JNK related kinase.



RESULTS

During the grant support period (7/97 to 6/00), we constructed a set of deletions to disrupt the potential regulatory domains of p53 on Gal4-p53(1-160) and on full-length p53 and obtained the following results:

Mutants Gal4-p53N(Δ92-109), Gal4-p53N(Δ116-127) and Gal4-p53N(Ser116/127Ala) had little effect on the putative inhibitory effect

We constructed a set of mutants, Gal4-p53N(Δ92-109), Gal4-p53N(Δ116-127) and Gal4-p53N(Ser116/127Ala), and examined their ability to activate transcription in vivo. This was performed using a transient transfection assay in COS-7 cells in which Gal4-p53 fusion proteins are tested for their ability to stimulate expression of luciferase gene under control of a promoter containing five Gal4 binding sites upstream of a TATA box. Gal4-p53N and Gal4-p53(1-92) were used as controls. Our result reveals that low levels of transcriptional activity of the mutant Gal4-p53N which are similar to Gal4-p53N, suggesting that mutants Gal4-p53N(Δ92-109), Gal4-p53N(Δ116-127) and Gal4-p53N(Ser116/127Ala) had little effect on the putative inhibition (Figure 1).

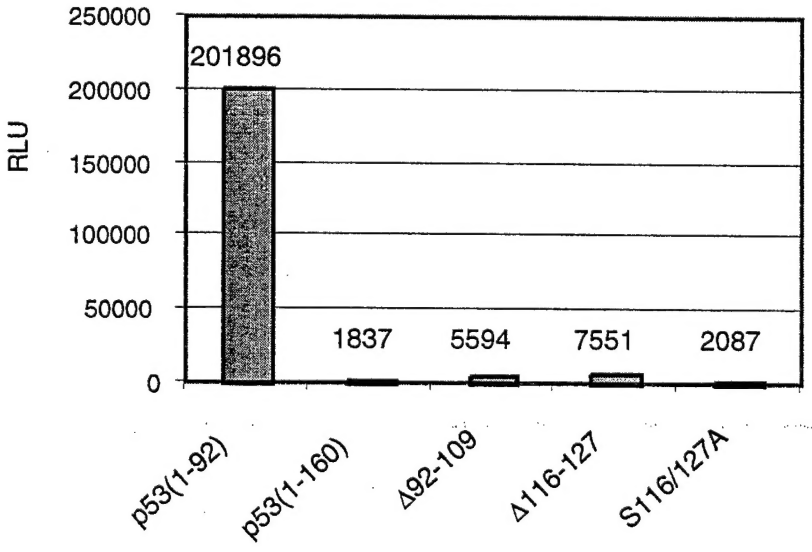


Figure 2. Transcriptional activity of Gal4-p53 constructs measure with a luciferase reporter plasmid in transfected COS-7 cells.

TPA stimulation inhibited the transcriptional activities of Gal4-p53N(Δ92-109) and Gal4-p53N(Δ116-127), but did not affect Gal4-p53(1-92).

We consider the possibility that failure to detect any effect for the mutants may be resulted from a lack of JNK or JNK related kinase activity in the cells. To test this, we stimulate the activity of JNK with TPA in NIH3T3 cells at different times after transfection. Our results show that stimulation by TPA reduced transcriptional activity of Gal4-p53N(Δ92-109) and Gal4-p53N(Δ117-128), but did not affect Gal4-p53(1-92), suggesting that residues within 92-109 and 117-128 domains may play a role to reduce p53 transcription activity in response to TPA stimulation. However, even with TPA stimulation, no significant effect on the inhibition was observed with deletion mutant Δ92-109 and Δ117-127.

Table I

	NO TPA	TPA STIMULATION
E4TATA-Luc	136	0
Gal4-p53N	0	0
p53N(1-92)	4236	5679
p53N(Δ92-109)	906	388
p53N(Δ116-127)	663	184
Gal4-VP16	83360	127590

Mutants p53(Δ92-109), p53(Δ116-127), and p53(S116/127D) significantly lost their ability to activate transcription in vivo.

To study the putative inhibitory domain, we also constructed a set of mutants, p53(Δ92-109), p53(Δ116-127) and p53(S116/127A) on full-length p53, and examined their ability to activate transcription in vivo. This was performed by using a transient transfection assay in SAO-S2 cells in which p53 proteins are tested for their ability to stimulate expression of luciferase gene under control of a promoter containing five p53 binding sites upstream of a TATA box. Results of representative luciferase assay are shown in Figure 3. This result reveals that mutation of serine to alanine residues [p53(S116A) and p53(S116/127A)] had little effect on p53 transcription activity, whereas mutation of serine to glutamate residues p53(S116/127D) lost its transcription activity. In addition, deletion mutants p53(Δ92-109) and p53(Δ116-127) also lost their transcription activity.

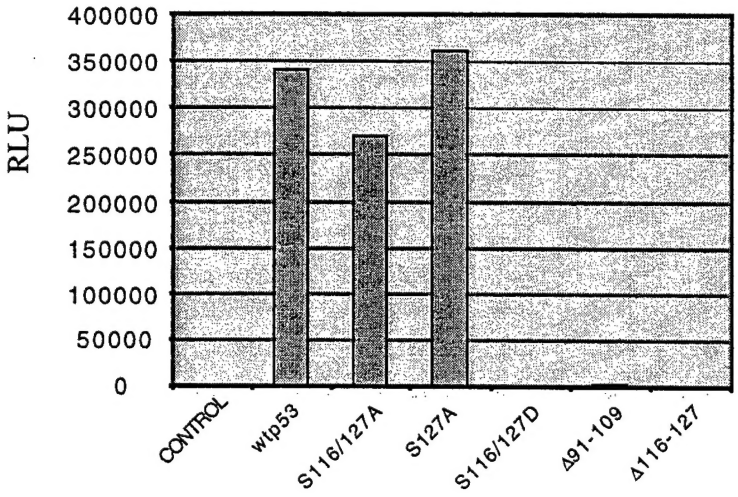


Figure 3. Transcriptional activity of pcDNAp53 constructs measured using a luciferase reporter plasmid in transfected SaoS-2 cells.

Wild-type p53 is rapidly degraded through the ubiquitin pathway. We consider the possibility that failure to detect any transcription activity may be caused by degradation of p53. To test this, we examined the p53 protein levels in Sao-S2 cells transiently transfected with wild-type p53 and mutant constructs. Results of a representative Western blot analysis are shown in Figure 4. Surprisingly, mutants p53(S116/127D) and p53(Δ92-109) displayed increased protein levels despite their defect in transcription. As concentrations of transcriptional activators have been shown regulated by the proteasome-mediated protein degradation, our results suggest that transcriptionally more active proteins may be more accessible to proteasome-mediated degradation.

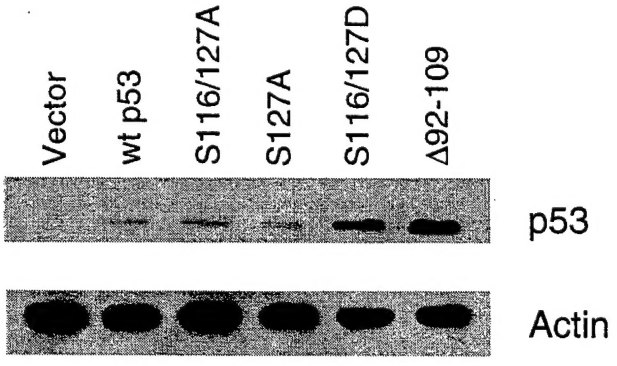


Figure 4. p53(S116/127D) and p53 (Δ92-109) display an increase in protein levels

Western blot analysis shows that the p53 protein levels in Sao-S2 cells transiently transfected with control, wild-type p53 and mutant constructs as indicated.

Mutants p53(Δ92-109) retained its abilities to bind to DNA, but p53(S116/127D) did not.

Next, we examined whether recruitment of p53 to promoter might enhance degradation by proteasome. To do this, we performed a gel shift experiment using nuclear extract from Sao-S2 cells transiently transfected with wild-type p53 and mutant constructs. Results of a representative gel-shift assay are shown in Figure 5. These results showed that the mutant p53(Δ92-109), but not p53(S116/127D), retained its abilities to bind to DNA, suggesting that the recruitment of p53 to promoter is not sufficient to protein degradation. We are currently investigated the molecular mechanism by which proteasome-mediated degradation correlates with transcription potency.

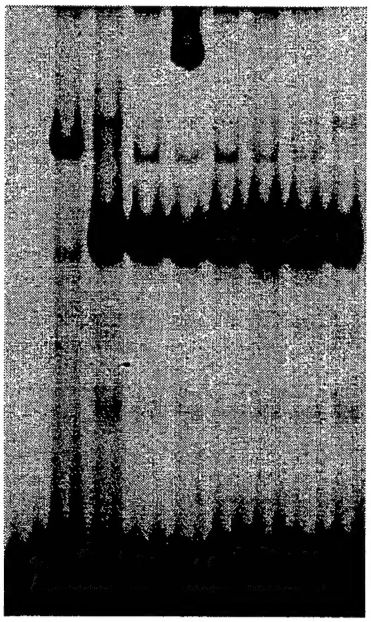


Figure 5. DNA-binding activities of mutant p53. A radiolabeled probe containing the p53-binding site from RGC was incubated with nuclear extracts from Sao-S2 cells transfected with wild-type p53 and mutant constructs as indicated. Lane 1 represents RGC probe, lane 2, 50 ng of purified p53, lane 3, transfected with control DNA, lane 4, transfected with wil-type p53, lane 5, lane 4 supershifted with anti-p53 antibody, lanes 6 to 9, transfected with Δ92, Δ117-127, S117/127A and S117/127D.

MAP-kinase is involved in the degradation of mutant p53 protein (Song et al, JBC)

Although we have failed to identify JNK or its related kinase as a kinase for wild-type p53, we have showed that MAP-kinase is involved in the degradation of mutant p53 protein. Overexpression of mutant p53 has been reported to promote tumorigenicity in several cancers. However, despite its potential importance, the signals regulating mutant p53 protein expression are not known. We have shown that a mutated form of p53 that is incapable of binding DNA is overexpressed in the acute promyelocytic leukemia NB4 cell line. Our results demonstrate that treatment of NB4 cells with bryostatin-1, which induces differentiation in this cell line, leads to

hyperphosphorylation of this DNA-binding impaired form of p53 via mitogen-activated protein kinase (MAPK). Following this phosphorylation, the p53 protein is degraded by the ubiquitin/proteasome pathway. Furthermore, we show that inhibition of p53 hyperphosphorylation by MAPK blocks p53 protein degradation and cell differentiation. In addition, inhibition of the ubiquitin/proteasome pathway also blocks p53 protein degradation and cell differentiation. These findings suggest a role for MAPK in the degradation of the DNA-binding impaired form of p53 protein and in the bryostatin-induced differentiation observed in this cell line. These results indicate the functional significance of p53 phosphorylation and degradation in cell differentiation.

KEY RESEARCH ACCOMPLISHMENTS

- MAPK is involved in regulating p53 transcription activity (Song et al, JBC 274:1677-1682)
- Serine residues 116 and 127 may play a role in stabilization of p53 protein levels

REPORTABLE OUTCOMES (PUBLICATIONS)

Song, X.D., H.M. Sheppard, A.W. Norman and X. Liu. 1999. MAP-kinase is involved in the degradation of p53 protein in the bryostatin-1 induced differentiation of the acute promyelocytic leukemia cell line NB4. *J. Biol. Chem.* **274**:1677-1682.

CONCLUSIONS

We have shown that two serine residues 116 and 127 may play a role in stabilization of p53 protein levels. Further experiments will be conducted to confirm these results. We have also shown that MAP-kinase is involved in the degradation of mutant p53 protein. Our findings suggest a role for MAPK in the degradation of mutated form of p53 protein and in cell

differentiation. These results were published as a JBC paper (Song, XD, H.M. Sheppard, A.W. Norman and X. Liu. 1999. Mitogen-activated protein kinase is involved in the degradation of p53 protein in the bryostatin-1 induced differentiation of the acute promyelocytic leukemia cell line NB4. J. Biol. Chem. 274:1677-1682).